Endocrine-disrupting chemicals can affect reproduction and development in humans and wildlife. We developed a computational model of the hypothalamic-pituitary-gonadal (HPG) axis in female fathead minnows to predict dose-response and time-course (DRTC) behaviors for endocrine effects of the aromatase inhibitor, fadrozole (FAD). The model describes adaptive responses to endocrine stress involving regulated secretion of a generic gonadotropin (LH/FSH) from the hypothalamic-pituitary complex. For model development, we used plasma 17β-estradiol (E2) concentrations and ovarian cytochrome P450 (CYP) 19A aromatase mRNA data from two time-course experiments, each of which included both an exposure and a depuration phase, and plasma E2 data from a third 4-day study. Model parameters were estimated using E2 concentrations for 0, 0.5, and 3 µg/l FAD exposure concentrations, and good fits to these data were obtained. The model accurately predicted CYP19A mRNA fold changes for controls and three FAD doses (0, 0.5, and 3 µg/l) and plasma E2 dose response from the 4-day study. Comparing the model-predicted DRTC with experimental data provided insight into how the feedback control mechanisms in the HPG axis mediate these changes; specifically, adaptive changes in plasma E2 levels occurring during exposure and “overshoot” occurring postexposure. This study demonstrates the value of mechanistic modeling to examine and predict dynamic behaviors in perturbed systems. As this work progresses, we will obtain a refined understanding of how adaptive responses within the vertebrate HPG axis affect DRTC behaviors for aromatase inhibitors and other types of endocrine-active chemicals and apply that knowledge in support of risk assessments.

Key Words: endocrine disruptors; biological modeling; nonmammalian species; dose response; biomarkers.

There is international concern regarding effects of endocrine-active environmental contaminants and commercial products on the health of humans and wildlife (Cooper and Kavlock, 1997; Daston et al., 2003; Hutchinson et al., 2006; Zacharewski, 1998). The dose-response and time-course (DRTC) behaviors of those chemicals are major determinants of health risk. In addition to adsorption, distribution, metabolism, and elimination, physiological adaptation (compensation) can affect DRTC behaviors. Characterization of adaptation is critical to modern toxicology as the field evolves from reliance on whole animal testing with apical endpoints toward predictive approaches anchored in understanding modes of action (National Research Council, 2007). The main goal of our current research was to develop a computational model of adaptive mechanisms in the hypothalamic-pituitary-gonadal (HPG) axis for a model vertebrate, the fathead minnow (Pimephales promelas).

Several computational models of the HPG axis have been described. For example, models have been developed to predict plasma sex-steroid concentrations and/or vitellogenin (egg yolk protein) concentrations in male fathead minnows exposed to estrogenic chemicals like 17α-ethinylestradiol or 17β-estradiol (E2) (Watanabe et al., 2009), in female fathead minnows exposed to 17α-ethinylestradiol or 17β-trenbolone (Li et al., 2011), and in female sciaenid fish exposed to PCBs and cadmium (Murphy et al., 2005). Kim et al. (2006) developed a computational model to describe normal functioning of the HPG axis in salmonids. Barton and Andersen (1998) developed a model of the HPG axis in rats to simulate hormone levels in testes and blood. HPG axis models for humans have been...
developed to describe changes in luteinizing hormone (LH) and testosterone (T) concentrations following treatment with the gonadotropin-releasing hormone (GnRH) agonist, triptorelin, and the GnRH receptor blocker, degarelix. (Tornøe et al., 2007), as well as the LH-releasing hormone antagonist, cetorelix (Pechstein et al., 2000).

To increase our understanding of mechanisms of compensation and recovery, we developed a computational model of the HPG axis in female fathead minnows to predict DRTC behaviors for effects of the aromatase inhibitor, fadrozole (FAD). FAD is a model endocrine-active chemical that competitively inhibits the steriodogenic enzyme, aromatase cytochrome P450 (CYP) 19A. Although FAD is not ecologically relevant, there are a variety of environmental contaminants that can inhibit aromatase activity and elicit similar effects (Petkov et al., 2009; Vinggaard et al., 2000). Our model includes a feedback regulatory loop within the HPG axis that mediates adaptive responses to endocrine-active chemical stressors by controlling the secretion of LH and follicle-stimulating hormone (FSH) from a generalized hypothalamic-pituitary complex.

In developing our model, we drew upon data from three separate experiments in which responses of female fathead minnows to FAD exposure were characterized. In the first study (experiment 1), fathead minnows were exposed to FAD via water at 0, 3, or 30 μg/l for 8 days followed by an 8-day recovery phase, with samples collected for various endpoints, including plasma E2 and ovarian CYP19A mRNA (Villeneuve et al., 2009). To increase our understanding of adaptive responses, an additional follow-up experiment was conducted (experiment 2, companion study). Fathead minnows were exposed to 0, 0.5, or 30 μg FAD/l for 8 days followed by 4, 8, 12, 16, or 20 days of recovery in control water, and various endpoints, including plasma E2 and ovarian CYP19A mRNA, were analyzed (Villeneuve et al., 2013—companion paper). Plasma E2 and ovarian CYP19A mRNA data from these two experiments, including recovery, were used for parameter estimation and model evaluation. Experimental design for experiments 1 and 2 are shown in Figure 1. To further evaluate our model, model predictions were tested against plasma E2 data from a third experiment (experiment 3) in which fathead minnows were exposed to 0, 0.04, 0.2, 1, or 5 μg FAD/l for 4 days (Ralston-Hooper et al., 2013).

The measurements from experiments 1 and 2 indicated adaptive changes (compensation) in plasma E2 levels during exposure, which resulted in a period of increased E2 production/concentrations, relative to controls, immediately following removal of the inhibitor (an overshoot), particularly at lower FAD test concentrations (Villeneuve et al., 2009). This type of behavior is not limited to FAD, but it is commonly observed with other endocrine-active chemicals that impact a variety of other pathways: inhibition of CYP11A, CYP17, 3β-hydroxysteroid dehydrogenase, and, even, agonism of the androgen receptor (Ankley et al., 2009, 2011, 2012; Ekman et al., 2011). Moreover, although our experimental model was a fish species, due to the significant conservation of the HPG axis structure and function, our model should be broadly extrapolatable to any vertebrate species. The model is intended to aid characterizing how regulatory feedback loops within the vertebrate endocrine axis mediate adaptive responses to endocrine-disrupting chemicals.

### MATERIALS AND METHODS

#### Mathematical model of HPG axis

The computational model of the HPG axis for FAD-exposed female fathead minnows was developed based on knowledge of biological mechanisms. The model consists of six tissue compartments: gill, brain (as a tissue including hypothalamic-pituitary complex), ovary, liver, venous blood, and rest of body (Figs. 2 and 3). The configuration of the compartments involved in HPG axis signaling and feedback control is consistent with the cardiovascular system of the exposed fish. However, because an arterial blood compartment is not essential for this model, it was not included. The model includes a generalized regulatory feedback loop that mediates adaptive responses to endocrine stress within the HPG axis. This negative regulatory loop controls the secretion of gonadotropins (LH and FSH). The time-varying concentrations of substrates are described by dynamic mass balances. We express the dynamic mass balance for the substrates in compartment y with volume V_y as

\[
V_y \frac{dC_{x,y}}{dt} = P_{x,y} - U_{x,y} - D_{x,y} + I_{x,y} - S_{x,y},
\]

where \( C_{x,y} \) is the concentration of substrate x in compartment y, \( P_{x,y} \) is the production rate of substrate x in compartment y, \( U_{x,y} \) is the utilization rate of substrate x in compartment y, \( D_{x,y} \) is the degradation rate of substrate x in compartment y, \( I_{x,y} \) is the import rate of substrate x into compartment y, and \( S_{x,y} \) is the secretion rate of substrate x from compartment y. The first two terms on the right side of Equation 1 represent the net metabolic reaction rate of substrate x. The last two terms represent the net uptake or release rate of substrate x in compartment y. The complete set of equations is provided in the Supplementary data. The model code can be obtained from RC at
The model for each tissue compartment is described in detail below.

**Gill compartment.** The gill serves as the major route for uptake of FAD from water by fish. There are no data on FAD uptake into skin. However, the rate of uptake into skin and from skin into the systemic circulation is likely to be much slower than via the gills because the gills provide a well-perfused, intimate interface between Tank water and fish blood. The computational model thus reflects this assumption that uptake via the gills is the main route of FAD entry into the fish, with skin being at most a minor route that is not quantitatively important. The gill compartment is broken into two subcompartments: gill lamellae and gill blood. The gill lamellae are gas-exchange units with walled, sac-like structures. FAD enters the gills via inspired water with uptake into the venous blood compartment, and FAD is removed from the gills via expired water with release from arterial blood. To determine the equilibrium concentration of FAD between water in the gill lamellae and gill blood, we assume a rapid equilibrium for FAD within water, with no metabolism of substrates is included in the gill compartment. The gills’ uptake and elimination of FAD are described by an algebraic expression for the mass balance of FAD entering and leaving the gills based on the method by Ramsey and Andersen (1984).

**Brain compartment.** We represent the brain-pituitary system as a generalized brain compartment. The brain-pituitary system communicates with hormone messengers, and endocrine glands produce and secrete the hormones to subsequent target tissues and glands. The brain initiates the process by sending neural signals. The hypothalamus responds to these neural signals by releasing GnRH, which is transported to the pituitary (Levavi-Sivan et al., 2010). In response to GnRH, the pituitary secretes LH and FSH into the blood that travel to the ovaries and initiates synthesis and secretion of hormones such as T and E2 via G protein-coupled receptor (GPCR) signaling. As part of a closed-loop negative feedback control system, specific hormones secreted by the ovaries (e.g., E2) are transported in the blood to the hypothalamus and pituitary to inhibit secretion of the initial hormone messengers (GnRH, LH, and FSH) (Chang et al., 2009; Levavi-Sivan et al., 2010; Trudeau, 1997).

Because empirical data for the peptide hormone messengers are unavailable, GnRH is not included in our model, and LH and FSH are represented as a generalized gonadotropin (LH/FSH) in our model. The model has zero-order synthesis, first-order release, and first-order degradation of LH/FSH in the brain compartment. The release of LH/FSH from the brain can be inhibited by E2 secreted from ovary (Chang et al., 2009; Levavi-Sivan et al., 2010; Trudeau, 1997). We model this negative feedback system, which controls the secretion of LH/FSH, by using the Hill function for a repressor with the Hill coefficient set to one. The E2 and FAD are imported into the brain compartment and secreted into the venous blood compartment by blood flow–limited kinetics.

**Ovary compartment.** The pituitary secretes LH and FSH into the blood that travels to the target ovary tissue, where LH and FSH bind to LH and FSH receptors to regulate steroidogenesis in the ovary. In our simplified model, LH/FSH in the venous blood compartment reversibly binds to LH/FSH receptors at the ovarian cell surface to control the synthesis of CYP19A mRNA, which in turn regulates E2 production in the ovary compartment. The model has zero-order synthesis of CYP19A mRNA (basal rate), and CYP19A mRNA synthesis is stimulated by LH/FSH–receptor complex. Synthesized CYP19A mRNA is translated to cyp19a (aromatase) by Michaelis-Menten kinetics. Both CYP19A mRNA and cyp19a are degraded in the ovary by first-order clearance.

Estradiol and FAD are absorbed into the ovary compartment and secreted into the venous blood compartment by flow–limited kinetics. Because intermediate steroid hormone concentrations are unavailable, we did not include a detailed metabolic pathway for steroidogenesis in the present model. Instead, the model includes the conversion of T into E2 by cyp19a and competitive inhibition of cyp19a by FAD. The concentration of T was set to a literature-reported constant value (Watanabe et al., 2007). The metabolism of T to E2 and the FAD inhibition of cyp19a are described by Michaelis-Menten kinetics for competitive inhibition.

**Liver compartment.** Estradiol and FAD are absorbed into the liver compartment and secreted into the venous blood compartment by blood flow–limited kinetics. The model has first-order degradation of E2 and FAD in the liver compartment.

**Venous blood compartment.** E2 and FAD from all the tissue compartments enter the venous blood compartment, as described by blood flow–limited kinetics, and are transported into the gill blood compartment. The LH/FSH are transported into the venous blood compartment and degraded by first-order kinetics. Because LH/FSH are proteins, they do not enter any tissues; however, they will bind to the LH/FSH receptors at the ovarian cell surface to stimulate the synthesis of CYP19A mRNA in the ovary compartment. Free LH/FSH, free LH/FSH receptors at the ovarian cell surface, and LH/FSH–receptor complex dynamics are described in the model.

**Rest of body compartment.** The rest of body compartment includes all tissues except gill, brain, ovary, and liver. E2 and FAD are absorbed into this compartment and secreted into the venous blood compartment by blood flow–limited kinetics.

**Parameter estimation.** The model utilizes physiological and biochemical parameters including tissue compartment volumes, blood flow rates, equilibrium partition coefficients, and biochemical reaction rates (i.e., transcription, translation, metabolism, transport, and degradation). Volumes of the major tissue compartments (ovary, liver, brain) and the whole body were measured in this study. The other physiological parameter values were calculated based on the literature (Table 1). Cardiac output and blood flows were based on measurements from rainbow trout (Nichols et al., 1990, 2004) and were allometrically scaled as described elsewhere (Li et al., 2011; Watanabe et al., 2009). However, cardiac output and blood flows were not scaled for differences in temperature (i.e., 1°C typical for rainbow trout vs. 25°C for fathead minnow). The equilibrium tissue/blood partition coefficients for E2 and blood/water and tissue/blood partition coefficients for FAD were assumed to be one based on our experimental results, which validated our model assumption that plasma concentrations of FAD were likely similar to water concentration. There were 21 biochemical parameters affecting the DRTC behaviors of CYP19A mRNA and E2 for the endocrine effects of FAD: five parameters were obtained from the literature, and 16 parameters were estimated using the mean E2 concentrations from fathead minnow studies. To capture adaptive changes in plasma E2 levels occurring during exposure and a subsequent “overshoot” behavior...
FIG. 3. Graphical representation of biochemical processes within the six compartments of the model: venous blood (A), gill blood (B), brain (C), ovary (D), liver (E), and rest of body (F). In the venous blood (A), processes include uptake and release of LH/FSH, E2, and FAD; degradation of LH/FSH. In the gill (B), processes include uptake and release of FAD and E2; LH/FSH synthesis, release, and degradation; E2-mediated inhibition of LH/FSH release into venous blood. In the brain (C), processes include uptake and release of FAD and E2; reversible binding of LH/FSH in blood to LH/FSH receptors on cell membrane (surface); LH/FSH receptor-mediated activation of CYP19A_mRNA synthesis; translation of CYP19A_mRNA into cyp19a; degradation of CYP19A_mRNA and cyp19a; conversion of T into E2 catalyzed by cyp19a; enzyme inhibition of cyp19a by FAD. In the liver (E), processes include uptake and release of E2 and FAD; degradation of E2 and FAD. In the rest of body (F), processes include uptake and release of FAD and E2.
TABLE 1
Physiological Constant for Weights, Volumes, Blood Flows, and Partition Coefficients (PC)

<table>
<thead>
<tr>
<th>Parameter description</th>
<th>Symbol</th>
<th>Compartment</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight of whole body</td>
<td>Wt_body</td>
<td>Whole body</td>
<td>9.451 × 10⁻⁵ kg</td>
<td>Measured</td>
</tr>
<tr>
<td>Volume of whole body</td>
<td>V_body</td>
<td>Whole body</td>
<td>9.470 × 10⁻ⁱ</td>
<td>Measured</td>
</tr>
<tr>
<td>Cardiac output</td>
<td>Q_cardiac</td>
<td>Whole body</td>
<td>1.110 × 10⁻¹ l/h</td>
<td>2.06 Wt_body⁰⁷ based on Nichols et al. (2004), Watanabe et al. (2009), Li et al. (2011)</td>
</tr>
<tr>
<td>Weight of gills</td>
<td>Wt_gill</td>
<td>Gill</td>
<td>1.578 × 10⁻³ kg</td>
<td>Assumed gill density is 1 kg/l (water density)</td>
</tr>
<tr>
<td>Volume of gills</td>
<td>V_gill</td>
<td>Gill</td>
<td>1.578 × 10⁻¹ l</td>
<td>Nichols et al. (1996)</td>
</tr>
<tr>
<td>Water flow through gills</td>
<td>Q_water_gill</td>
<td>Gill</td>
<td>5.714 × 10⁻¹ l/h</td>
<td>10.6 Wt_body⁰⁷ based on Nichols et al. (2004), Watanabe et al. (2009), Li et al. (2011)</td>
</tr>
<tr>
<td>Weight of ovary</td>
<td>Wt_ovary</td>
<td>Ovary</td>
<td>1.488 × 10⁻⁴ kg</td>
<td>Measured</td>
</tr>
<tr>
<td>Volume of ovary</td>
<td>V_ovary</td>
<td>Ovary</td>
<td>1.440 × 10⁻¹ l</td>
<td>Weight/density (1.02 kg/l) from Breen et al. (2007)</td>
</tr>
<tr>
<td>Blood flow to ovary</td>
<td>Q_ovary</td>
<td>Ovary</td>
<td>6.292 × 10⁻¹ l/h</td>
<td>3.6 Q_cardiac(Wt_ovary/Wt_body) based on Nichols et al. (1990), Watanabe et al. (2009), Li et al. (2011)</td>
</tr>
<tr>
<td>Weight of liver</td>
<td>Wt_liver</td>
<td>Liver</td>
<td>2.305 × 10⁻³ kg</td>
<td>Assumed</td>
</tr>
<tr>
<td>Volume of liver</td>
<td>V_liver</td>
<td>Liver</td>
<td>2.400 × 10⁻¹ l</td>
<td>Measured</td>
</tr>
<tr>
<td>Blood flow to liver</td>
<td>Q_liver</td>
<td>Liver</td>
<td>6.500 × 10⁻¹ l/h</td>
<td>2.4 Q_cardiac(Wt_liver/Wt_body) based on Nichols et al. (1990), Watanabe et al. (2009), Li et al. (2011)</td>
</tr>
<tr>
<td>Weight of brain</td>
<td>Wt_brain</td>
<td>Brain</td>
<td>1.004 × 10⁻⁵ kg</td>
<td>Assumed</td>
</tr>
<tr>
<td>Volume of brain</td>
<td>V_brain</td>
<td>Brain</td>
<td>1.200 × 10⁻¹ l</td>
<td>Measured</td>
</tr>
<tr>
<td>Blood flow to brain</td>
<td>Q_brain</td>
<td>Brain</td>
<td>4.247 × 10⁻¹ l/h</td>
<td>3.6 Q_cardiac(Wt_brain/Wt_body) based on Nichols et al. (1990), Watanabe et al. (2009), Li et al. (2011)</td>
</tr>
<tr>
<td>Weight of venous</td>
<td>Wt_venous</td>
<td>Venous blood</td>
<td>2.448 × 10⁻⁵ kg</td>
<td>Nichols et al. (1996), Robinson et al. (1992)</td>
</tr>
<tr>
<td>Volume of venous</td>
<td>V_venous</td>
<td>Venous blood</td>
<td>2.448 × 10⁻¹ l</td>
<td>Weight/density of water (1 kg/l)</td>
</tr>
<tr>
<td>Volume of rest of body</td>
<td>V_RoB</td>
<td>Rest of body</td>
<td>7.267 × 10⁻¹ l</td>
<td>V_body – Volume of other organs</td>
</tr>
<tr>
<td>Blood flow to rest of body</td>
<td>Q_RoB</td>
<td>Rest of body</td>
<td>3.738 × 10⁻¹ l/h</td>
<td>Q_cardiac – Blood flow to other organs</td>
</tr>
<tr>
<td>Water:blood PC for FAD</td>
<td>P_FAD_water: blood</td>
<td>Gill</td>
<td>1</td>
<td>Assumed</td>
</tr>
<tr>
<td>Ovary:blood PC for FAD</td>
<td>P_FAD_ovary: blood</td>
<td>Ovary</td>
<td>1</td>
<td>Assumed</td>
</tr>
<tr>
<td>Liver:blood PC for FAD</td>
<td>P_FAD_liver: blood</td>
<td>Liver</td>
<td>1</td>
<td>Assumed</td>
</tr>
<tr>
<td>Brain:blood PC for FAD</td>
<td>P_FAD_brain: blood</td>
<td>Brain</td>
<td>1</td>
<td>Assumed</td>
</tr>
<tr>
<td>Rest of body:blood PC for FAD</td>
<td>P_FAD_RoB: blood</td>
<td>Rest of body</td>
<td>1</td>
<td>Assumed</td>
</tr>
</tbody>
</table>

Parameters were estimated with a nonlinear optimization algorithm using MATLAB R2010a (Mathworks, Natick, MA) software. The Nelder-Mead simplex method was used due to its relative insensitivity to the initial parameter values compared with other common methods, such as Newton’s method, and its robustness to discontinuities (Nelder and Mead, 1965).

Sensitivity analysis. To examine model parameter uncertainty, we performed a sensitivity analysis on 25 parameters: 6 cardiovascular parameters in Table 1 and 19 biochemical parameters in Tables 2 and 3. The sensitivity function relates changes of the model output (plasma E2 concentrations and function relates changes of the model output (plasma E2 concentrations and occurring postexposure for 3 µg/l FAD dose, model parameters were estimated using E2 concentrations for 0, 0.5, and 3 µg/l FAD doses. The CYP19A mRNA data were not used to estimate model parameters because we validated our model predictions using this dataset. We incorporated measured data from both experiments described above in both parameter estimation and model evaluation.

Using the least squares method, eight parameters in the ovary compartment (k₀_syn_mRNA, Kᵢ_syn_mRNA, kᵢ_syn_mRNA), two parameters in the liver compartment (k₀_FAD, Kᵢ_FAD), and four parameters in the blood compartment (k₀_FAD, Kᵢ_FAD, kᵢ_syn_mRNA), were estimated with the time-course data of E2 from the fathead minnow studies. We let nᵢ be the number of time points in the time-course data of E2 for the i th FAD dose (including control): ĈE2blood be the measured E2 plasma concentrations for the i th FAD dose at the j th time; CᵢE2blood(t; C′ FAD, k) be the model-predicted concentrations of E2 in the venous blood compartment at the j th time, tᵢ for the i th FAD dose (including control), C′ᵢ, with parameter set

for d = 1, 2, 3, and i = 1, ..., nᵢ. Then, the least squares estimate,

\[ \hat{\theta} = (C₀_syn_mRNA, Kᵢ_syn_mRNA, kᵢ_syn_mRNA, Vᵢ_syn_mRNA, Kᵢ_syn_mRNA, kᵢ_syn_mRNA, Kᵢ_FAD, Kᵢ_FAD, kᵢ_syn_mRNA, kᵢ_syn_mRNA, Kᵢ_FAD, Kᵢ_FAD, kᵢ_syn_mRNA, kᵢ_syn_mRNA, Kᵢ_FAD, Kᵢ_FAD, kᵢ_syn_mRNA, kᵢ_syn_mRNA, Kᵢ_FAD, Kᵢ_FAD, kᵢ_syn_mRNA, kᵢ_syn_mRNA, Kᵢ_FAD, Kᵢ_FAD, kᵢ_syn_mRNA, kᵢ_syn_mRNA, Kᵢ_FAD, Kᵢ_FAD, kᵢ_syn_mRNA, kᵢ_syn_mRNA) \]

is the parameter values \( \hat{\theta} \), which minimizes the cost function

\[ J(\theta) = \frac{1}{2} \sum_{i=1}^{i=n} (CᵢE2blood(t; C′ FAD, k) - CᵢE2blood(t; C′ FAD, k))^2. \] (2)

Parameters were estimated with a nonlinear optimization algorithm using MATLAB R2010a (Mathworks, Natick, MA) software.
ADAPTIVE RESPONSES TO ENDOCRINE DISRUPTION

Parameter description | Symbol | Compartment | Value | Reference
--- | --- | --- | --- | ---
Catalysis rate for CYP19A | $k_{cat,CYP19A}$ | Ovary | $6.0993 \times 10^{-4}$/h | Zhao et al. (2001)
Michaelis constant for T | $K_m,T$ | Ovary | $9.250 \times 10^{-6}$/µmol | Zhao et al. (2001)
Concentration of T | $C_T$ | Ovary | $1.4146 \times 10^{-2}$/µmol/l | Zhao et al. (2001)
Total amount of LH/FSH receptor | $A_{LDHFSH,ovary}$ | Ovary | $2.88 \times 10^{-2}$/µmol | Miwa et al. (1994)
Binding rate of LH/FSH (blood) to LH/FSH receptor (ovary) | $k_{LHFSH,ovary}$ | Venous blood | $0.2 \times 10^{-5}$(µmol/h) | Watanabe et al. (2009)

### Fixed Biochemical Parameters

<table>
<thead>
<tr>
<th>Parameter description</th>
<th>Symbol</th>
<th>Compartment</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Basal synthesis rate of CYP19A mRNA | $k_{syn,mRNA,basal}$ | Ovary | $1.474 \times 10^{-1}$/µmol/h | Zhao et al. (2001)
| Maximum synthesis rate of CYP19A mRNA | $k_{syn,mRNA,max}$ | Ovary | $9.250 \times 10^{-6}$/µmol | Zhao et al. (2001)
| Degradation rate of CYP19A mRNA | $k_{deg,mRNA}$ | Ovary | $1.494 \times 10^{-7}$/h | Zhao et al. (2001)
| Michaelis constant for synthesis of CYP19A | $K_{m,syn,mRNA}$ | Ovary | $3.149 \times 10^{-4}$/µmol | Zhao et al. (2001)
| Degradation rate of CYP19A | $h_{deg,CYP19A}$ | Ovary | $1.374 \times 10^{-2}$/µmol | Zhao et al. (2001)
| Inhibition constant of FAD | $K_{i,FAD}$ | Ovary | $1.981 \times 10^{-1}$/µmol | Zhao et al. (2001)
| Activation constant for synthesis of CYP19A mRNA | $k_{syn,mRNA}$ | Ovary | $2.278 \times 10^{-5}$/µmol/h | Zhao et al. (2001)
| Degradation rate of FAD | $k_{deg,FAD}$ | Liver | $4.698 \times 10^{-1}$/h | Zhao et al. (2001)
| Degradation rate of E2 | $k_{deg,E2}$ | Liver | $1.167 \times 10^{-1}$/h | Zhao et al. (2001)
| Zero-order synthesis rate of LH/FSH | $V_{0,synth,CYP19A}$ | Ovary | $6.537 \times 10^{-4}$/µmol | Zhao et al. (2001)
| Degradation rate of LH/FSH | $h_{deg,LHFSH}$ | Ovary | $1.374 \times 10^{-2}$/µmol | Zhao et al. (2001)
| Releasing rate of LH/FSH | $k_{LHFSH,ovary}$ | Ovary | $1.534 \times 10^{-2}$/µmol | Zhao et al. (2001)
| Inhibition constant of E2 | $K_{i,E2}$ | Liver | $3.407 \times 10^{-1}$/h | Zhao et al. (2001)
| Releasing rate of LH/FSH (blood) from LH/FSH receptor (ovary) | $k_{LHFSH,ovary,h}$ | Venous blood | $8.004 \times 10^{-1}$/h | Zhao et al. (2001)
| Degradation rate of LH/FSH | $k_{deg,LHFSH}$ | Venous blood | $1.831 \times 10^{-1}$/h | Zhao et al. (2001)

Table 2: Estimated Parameters

### Estimated Parameters

<table>
<thead>
<tr>
<th>Parameter description</th>
<th>Symbol</th>
<th>Compartment</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Basal synthesis rate of CYP19A mRNA | $k_{syn,mRNA,basal}$ | Ovary | $1.474 \times 10^{-1}$/µmol/h | Zhao et al. (2001)
| Maximum synthesis rate of CYP19A mRNA | $k_{syn,mRNA,max}$ | Ovary | $9.250 \times 10^{-6}$/µmol | Zhao et al. (2001)
| Degradation rate of CYP19A mRNA | $k_{deg,mRNA}$ | Ovary | $1.494 \times 10^{-7}$/h | Zhao et al. (2001)
| Michaelis constant for synthesis of CYP19A | $K_{m,syn,mRNA}$ | Ovary | $3.149 \times 10^{-4}$/µmol | Zhao et al. (2001)
| Degradation rate of CYP19A | $h_{deg,CYP19A}$ | Ovary | $1.374 \times 10^{-2}$/µmol | Zhao et al. (2001)
| Inhibition constant of FAD | $K_{i,FAD}$ | Ovary | $1.981 \times 10^{-1}$/µmol | Zhao et al. (2001)
| Activation constant for synthesis of CYP19A mRNA | $k_{syn,mRNA}$ | Ovary | $2.278 \times 10^{-5}$/µmol/h | Zhao et al. (2001)
| Degradation rate of FAD | $k_{deg,FAD}$ | Liver | $4.698 \times 10^{-1}$/h | Zhao et al. (2001)
| Degradation rate of E2 | $k_{deg,E2}$ | Liver | $1.167 \times 10^{-1}$/h | Zhao et al. (2001)
| Zero-order synthesis rate of LH/FSH | $V_{0,synth,CYP19A}$ | Ovary | $6.537 \times 10^{-4}$/µmol | Zhao et al. (2001)
| Degradation rate of LH/FSH | $h_{deg,LHFSH}$ | Ovary | $1.374 \times 10^{-2}$/µmol | Zhao et al. (2001)
| Releasing rate of LH/FSH | $k_{LHFSH,ovary}$ | Ovary | $1.534 \times 10^{-2}$/µmol | Zhao et al. (2001)
| Inhibition constant of E2 | $K_{i,E2}$ | Liver | $3.407 \times 10^{-1}$/h | Zhao et al. (2001)
| Releasing rate of LH/FSH (blood) from LH/FSH receptor (ovary) | $k_{LHFSH,ovary,h}$ | Venous blood | $8.004 \times 10^{-1}$/h | Zhao et al. (2001)
| Degradation rate of LH/FSH | $k_{deg,LHFSH}$ | Venous blood | $1.831 \times 10^{-1}$/h | Zhao et al. (2001)

### Mathematical Model of HPG Axis

The physiological constants for tissue weights, tissue volumes, blood flows, and partition coefficients are shown in

**RESULTS**

**FAD Exposure Data**

Exposure to 0.5 µg FAD/l had no considerable effect on either E2 concentrations or CYP19A mRNA expression (Fig. 4). For the 3 µg/l FAD group, E2 concentrations were greatly reduced after 2 days of exposure but recovered to the control level between 2 and 8 days of exposure, exceeded those of controls at 1 day postexposure, and returned to control levels after 2 days postexposure (Fig. 4A). Expression of CYP19A mRNA was greatly elevated after 4 or 8 days of exposure and returned to control levels postexposure period (Fig. 4B). As one would predict, exposure to 30 µg FAD/l had the most considerable effect on E2 concentrations and CYP19A mRNA abundance. The E2 concentrations were greatly reduced within 1 day of exposure to FAD, remained greatly reduced throughout the exposure period, and then rebounded back to the control levels after 2 days exposure (Fig. 4A). Expression of CYP19A mRNA was greatly elevated at 2 days of exposure and thereafter, but returned to control levels during depuration (Fig. 4B).

---

The ovarian expression of CYP19A mRNA (to changes in the model parameters. We calculated relative sensitivity functions $\frac{\partial E_{2,blood}}{\partial k_i}$ and $\frac{\partial C_{mRNA,ovary}}{\partial k_i}$ with respect to parameter $k_i$ for each of the model-predicted concentrations $E_{2,blood}$ and fold changes of CYP19AmRNA in the ovary compartment $C_{mRNA,ovary}$, respectively, as described by

$$ R_{E2,blood}(t) = \left( \frac{k}{C_{E2,blood}(t)} \right) \frac{\partial C_{E2,blood}(t)}{\partial k_i} $$

and

$$ R_{C_{mRNA,ovary}}(t) = \left( \frac{k}{F_{E2,blood}(t)} \right) \frac{\partial F_{E2,blood}(t)}{\partial k_i} $$

MATLAB was used to numerically calculate the partial derivatives in $R_{E2,blood}(t)$ and $R_{C_{mRNA,ovary}}(t)$ (Equations 3 and 4) for control and each FAD dose. To rank the relative sensitivities, we calculated the $L^2$ norm across time for each relativity sensitivity function as described by

$$ L^2 \text{ norm}(R_{E2,blood}(t)) = \sqrt{\int R_{E2,blood}(t)^2 \, dt} $$

and

$$ L^2 \text{ norm}(R_{C_{mRNA,ovary}}(t)) = \sqrt{\int R_{C_{mRNA,ovary}}(t)^2 \, dt} $$

Magnitudes of the relative sensitivities relate the degree to which changes in parameter values lead to changes in model outputs. The sensitivity analysis orders the inputs by importance, identifying the main contributors to the variation in the model outcome. Parameters with high sensitivity are more important for the model output than parameters with low sensitivity.
The five literature-derived biochemical parameters and the biochemical parameters estimated by fitting the model predictions to the measured mean plasma E2 concentrations for 0, 0.5, and 3 µg/l FAD doses are shown in Tables 2 and 3, respectively. The time for convergence to the solution for the nonlinear parameter estimation was typically around 3 h on an Intel Core 2 Duo processor using MATLAB.

We compared the model-predicted concentrations of venous E2 with time-course measurements of plasma E2. For the FAD doses used for parameter estimation (0, 0.5, and 3 µg/l), the model-predicted E2 concentrations correspond closely to the mean time-course measurements (Figs. 5A–C). For the 3 µg FAD/l treatment, the model captured the plasma E2 compensation during exposure and the overshoot and return to control level postexposure (Fig. 5C). For the high FAD dose (30 µg/l), which was not included for parameter estimation, the model-predicted E2 concentrations during exposure also correspond closely to the mean time-course measurements (Fig. 5D). However, the model overpredicted E2 concentrations postexposure (Fig. 5D).

Figure 6A shows the modeled venous E2 dose response during FAD exposure on days 1, 4, and 8.

**Table 1.** Measurements of plasma E2 (A) and ovary CYP19A mRNA (B) for control and three FAD concentrations (0.5, 3, and 30 µg/l). Measurements (mean ± SD) of E2 concentrations and CYP19A mRNA fold changes relative to controls were plotted from two experiments: control data includes four sampling times during exposure and seven sampling times postexposure; 0.5 µg/l—two sampling times during exposure and five sampling times postexposure; 3 µg/l—four sampling times during exposure and four sampling times postexposure; 30 µg/l—four sampling times during exposure and seven sampling times postexposure. n = 16 for most conditions (combination of treatment and time point) with two experiments (control and FAD concentration of 30 µg/l at d1, d8, d12, and d16) and n = 8 for most conditions with one experiment. Vertical dashed line indicates period of FAD exposure. This figure can be viewed in color online.
having the lowest venous E2 concentration and day 8 having the highest venous E2 concentration. Additionally, the model predictions are plotted as a function of FAD concentration and time for venous E2 concentrations (A–D) and ovary CYP19A mRNA fold changes relative to controls (E–H). Model predictions were compared with measurement data from two experiments: control data include four sampling times during exposure and seven sampling times postexposure; 0.5 µg/l—two sampling times during exposure and five sampling times postexposure; 3 µg/l—four sampling times during exposure and four sampling times postexposure; 30 µg/l—four sampling times during exposure and seven sampling times postexposure. Vertical dashed lines indicate period of FAD exposure.

For model validation, we compared the model-predicted and measured ovary CYP19A mRNA changes. Although the measurements were not used for parameter optimization, measured ovary CYP19A mRNA fold changes correspond well to the time-course data for control and the three FAD doses (Figs. 5E–H). Figure 6B shows the modeled ovary cyp19a dose response during FAD exposure on days 1, 4, and 8. The model predictions monotonically increased across dose, with
FIG. 6. Modeled dose response during FAD exposure. Model predictions were plotted as a function of FAD concentrations for venous E2 concentrations (A,C) and ovary CYP19A mRNA fold changes relative to controls (B) during exposure to FAD on days 1, 4, and 8. For day 4 (C), measured venous E2 concentrations for five FAD concentrations (0, 0.04, 0.2, 1, and 5 µg/l) with n = 11 or 12 were plotted with model predictions. Same model predictions are shown in (A) and (C) for FAD concentrations between 0 and 5.5 µg/l.
FAD exposure on day 4 having the highest ovary CYP19A mRNA and day 1 having the lowest ovary CYP19A mRNA. Additionally, the model predictions are plotted as a function of FAD concentration and time for ovary CYP19A mRNA fold changes (Supplementary fig. S1B).

To further validate our model, we tested model predictions against plasma E2 data from the 4 days exposure study that was not included in our model development. The model-predicted dose-response curve for venous E2 corresponds well to plasma E2 measurements, even with all five FAD doses (0, 0.04, 0.2, 1, and 5 µg FAD/l) different from the FAD doses used for model calibration (Fig. 6C).

Sensitivity Analysis

Figs. 7A, B and 8A, B show the relative sensitivities for modeled E2 and CYP19A mRNA, respectively, plotted as a function of the 19 biochemical model parameters for control and three FAD test concentrations (0.5, 3, and 30 µg/l) during exposure and postexposure, respectively. Overall, E2 (Figs. 7A and B) and CYP19A mRNA (Figs. 8A and B) were highly to
moderately sensitive to the model parameters during exposure and postexposure, except for a few parameters ($k_{\text{syn_mRNA,basal}}$, $k_{\text{loss_FAD}}$, $k_{\text{loss_LHFSH}}$).

Supplementary figures 2SA,B and 3SA,B show the relative sensitivities for modeled E2 and CYP19A mRNA, respectively, plotted as a function of the six cardiovascular model parameters for control and three FAD test concentrations (0.5, 3, and 30 µg/l) during exposure and postexposure, respectively. Both E2 (Supplementary fgs. 2A and B) and CYP19A mRNA (Supplementary fgs. 3A and B) were most sensitive to cardiac output ($Q_{\text{cardiac}}$) and highly sensitive to blood flow to ovary ($Q_{\text{ovary}}$) and rest of body ($Q_{\text{RoB}}$) during exposure and postexposure.

**DISCUSSION**

To predict the DRTC behaviors for endocrine effects of the aromatase inhibitor, FAD, we developed a mechanistic mathematical model and estimated biochemical parameters for the HPG
axis in female fathead minnows. Experimental gene expression and steroid data were used to develop the computational model. To examine the adaptive responses to FAD, a regulatory feedback loop within the HPG axis that can mediate adaptive responses to endocrine-active chemicals was included in the model. Sensitivity analysis indicates that the regulatory feedback loop plays an important role in our model because E2 and CYP19A mRNA were highly to moderately sensitive to the parameters ($k_{LHFSH}$, $K_{E2}$) associated with the feedback control loop. The model closely fits the dynamic E2 concentrations for baseline, 0.5, and 3 μg/l FAD test groups. The model also predicts dynamic CYP19A mRNA fold changes for baseline and three (0.5, 3, and 30 μg/l) FAD concentrations and venous E2 dose response during FAD exposure (0, 0.04, 0.2, 1, and 5 μg FAD/l) on day 4.

Our HPG axis model with one negative feedback loop was capable of simulating several important DRTC phenomenon observed empirically. The model predicts declining estradiol concentrations following initial exposure to FAD. Through the feedback loop incorporated into the model, declining E2 concentrations stimulate increased release of a generalized gonadotropin (LH/FSH), leading to increased transcription of CYP19A mRNA and an assumed parallel increase in aromatase activity. The increased aromatase transcription and activity confronted with a constant level of inhibition lead to a modest increase in the predicted venous E2 concentrations over an 8-day period of exposure, which is particularly evident at the 3 μg/l FAD dose. Although the model slightly underpredicts the magnitude of increased E2 production over the course of the exposure period, the general behavior of the model prediction was consistent with that observed empirically. Immediately after chemical removal, the model successfully predicts a brief period over which circulating E2 concentrations in the FAD-treated fish exceed those of controls before gradually returning to normal levels, as CYP19A mRNA transcription and assumed activity decline back to baseline levels. Although ovarian CYP19A mRNA data and plasma E2 data from the 4-day exposure study were not included in model development, the model also captures dynamic CYP19A mRNA fold changes and venous E2 dose response during FAD exposure on day 4. Therefore, our mechanistic model provides a reasonable simulation of critical DRTC behaviors observed in vivo.

There are some limitations to our model based on the model structure and assumptions and data available for model evaluation. First, although our feedback mechanism results in a gradual increase in venous E2 concentrations over the course of exposure at the 3 μg/l FAD dose, the model underpredicts the magnitude of compensation observed in vivo. This could suggest, for instance, that the current model underestimates the degree of increase in aromatase activity that is currently predicted from a specific fold-change increase in CYP19A mRNA transcription. This is a relationship that could be investigated via additional time-course experiments, as methods to measure both aromatase activity and CYP19A mRNA transcripts are well developed (Villeneuve et al., 2006). Other parameters such as the rate and magnitude of feedback response per unit change in circulating E2 may also play a role, but the precise mechanisms of the feedback response are not well understood nor can specific measurements of gonadotropin peptide concentrations be made readily for cyprinid fish species like the fathead minnow. Similarly, it is recognized that the ovary of an asynchronous spawning fish species, like fathead minnow, contains a heterogeneous collection of oocytes and ovarian follicles at multiple different stages of development. It is expected that these subpopulations of ovarian cells likely have differential sensitivity or capacity to respond to endocrine feedback (e.g., mediated by FSH/LH); thus, changes in the overall distribution of these different populations in the ovary could also influence the relative magnitudes of response. However, most experiments to date have focused data collection on the tissue level of resolution. Data collected at the level of specific subpopulations of oocytes and ovarian follicles within the ovary are lacking. Second, although our feedback mechanism results in postexposure “overshoot” in venous E2 concentrations at the 30 μg/l FAD dose, the model overpredicts the magnitude of postexposure “overshoot” that was observed in vivo. It is likely that there are additional biological phenomena operating in vivo that impose limits on the overall magnitude/strength of the compensatory response and the corresponding “overshoot” that occurs when the inhibitor is abruptly removed from the system. Thus, we explored whether the compensatory response may be limited by electron transfer because aromatase and other microsomal type II cytochrome P450 enzymes depend on electron transfer from P450 oxidoreductase for their function (Miller, 2005). We hypothesized that unless the redox partners for aromatase were also induced as part of a compensatory response, electron transfer efficiency could become the limiting rate of estradiol synthesis, even if aromatase transcription and presumably translation continued to increase. We extended our model to evaluate whether such a mechanism could improve the ability of the model to fit the empirical data; however, that was not the case. (Dynamic molecular balance equations for the extended model are provided in the Supplementary data.) Another possible explanation is that E2 production becomes substrate limited. Specifically, T is the C19 steroid precursor that undergoes aromatization to form E2. Within the ovary, T production is localized within the theca cells, whereas aromatization to E2 takes place in the granulosa cells (Yaron, 1995). Some of the key proteins that are rate limiting for T production include StAR and CYP11A (Miller, 1988; Stocco, 2001). It is notable that whereas targets thought to be directly involved in stimulating E2 production by granulosa cells (cyp19a and FSHR) were robustly induced in both experiments (Villeneuve et al., 2009, 2013—companion paper), CYP11A and STAR expression was either transiently induced (Villeneuve et al., 2009) or not at all (Villeneuve et al. 2013—companion paper). This suggests the possibility that the availability of T could impose limits on the degree of compensatory E2 production that could be achieved by increasing cyp19a transcription and translation. In the present experiments, plasma volumes were insufficient to allow for quantification of both E2 and T.
To better understand adaptive responses to endocrine stress, we further investigated another potential feedback regulatory loop within the HPG axis. Based on the observation of an apparent tight, parallel coupling between plasma E2 concentrations and CYP19A mRNA expression in fish exposed to 30 μg FAD/l, we hypothesized an autocrine feedback whereby the internalization of LH/FSH receptors could be stimulated by E2 in ovary to provide a more rapid feedback response to alterations in E2 synthesis rates. This hypothesis was based on the knowledge that many GPCRs go through cycling to and from the cell membrane (McArdle et al. 2002). There is sufficient characterization to support the idea that LHR and FSHR undergo this type of cycling (Krishnamurthy et al., 2003). Therefore, we extended our model to include such an additional autocrine feedback loop, which regulates LH and FSH receptor recycling in the ovary. (Dynamic molecular balance equations for the extended model are provided in the Supplementary data.) We hypothesized that the LH/FSH receptors cycle between the ovarian cell surface and cytosol, and the receptors must be at the cell surface to be activated by reversible binding of LH/FSH. However, the extended model with an additional feedback regulatory loop did not improve the model fit for plasma E2 or ovary CYP19A mRNA considerably. This is another example of how the model can be used as a preliminary hypothesis testing tool before proceeding with more expensive and time-consuming experimentation.

There are several potential extensions and applications for our model. First, investigations of other regulatory mechanisms for different test concentrations/doses could be guided by this work. For example, in future studies, we plan to investigate other possible regulatory mechanisms for higher treatment groups using genomic (e.g., microarray) technologies in combination with targeted experiments to quantitatively define the relationships among key events. Second, the model can be extended to endocrine-active chemicals that impact other pathways, including endocrine-active chemicals with multiple modes of action. For instance, in future studies, we plan to extend our model to other aromatase inhibitors with multiple modes of action, such as Prochloraz, an antifungal pesticide with relevance for human and ecological risk assessments. Thus, our approach can be expanded to other chemicals that interact with the HPG axis to ultimately provide a generic capability for generating useful predictions of DRTC for disruptions of the HPG axis in fish. Third, our fish model could be modified for other vertebrate species including humans. The endocrine system, which originated during the early evolution of fish, is highly conserved across vertebrate species. Hence, the components of the endocrine system are fundamentally the same for all vertebrates. With the appropriate in vivo aromatase inhibition data from other vertebrate species, our model could be modified and reparameterized. Because the modeling approach taken is extendable to other chemicals that interact with the HPG axis and other vertebrate species, it could, with substantial further development, provide a generic capability for generating useful predictions of DRTC for disruptions of the HPG axis for both human and ecological risk assessments.

Comparing model-predicted DRTC with experimental data provided insight into how the feedback control mechanisms embedded in the HPG axis mediate the dynamic dose-response changes. This study demonstrates the value of mechanistic computational modeling to examine and predict the possible dynamic behaviors and to formulate and test hypotheses. As this work progresses, we will obtain a refined understanding of how adaptive responses within the vertebrate HPG axis can affect DRTC behaviors for aromatase inhibitor and other types of endocrine-active chemicals and apply that knowledge in support of risk assessments.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

ACKNOWLEDGMENTS

We thank Hisham El-Masri and Richard Judson for their review comments and helpful suggestions. Although this manuscript was reviewed by the U.S. Environmental Protection Agency and approved for publication, it may not reflect official Agency policy. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

REFERENCES


